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MANUAL OF METHODS IN AQUATIC ENVIRONMENT RESEARCH

Part 6 – Toxicity Tests

with the cooperation of the
United Nations Environment Programme

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS

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UNITED NATIONS ENVIRONMENT PROGRAMME

MANUAL OF METHODS IN AQUATIC ENVIRONMENT RESEARCH
Part 6. Toxicity Tests

by

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FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
Rome, 1982

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PREPARATION OF THIS MANUAL

This manual was prepared as part of a cooperative project of the United Nations Environment Programme entitled:

Effects of Pollution on Living Aquatic Resources and Scientific Basis for Monitoring

with the Food and Agriculture Organization of the United Nations as cooperating agency.

DEFINITION OF MARINE POLLUTION

Pollution of the marine environment means: "The introduction by man, directly or indirectly, of substances or energy into the marine environment (including estuaries) which results in such deleterious effects as harm to living resources, hazards to human health, hindrance to marine activities including fishing, impairment of quality for use of sea water and reduction of amenities".

IMCO/FAO/Unesco/WMO/WHO/IAEA/UN/UNEP Joint Group of Experts on the Scientific Aspects of Marine Pollution (GESAMP)

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PREFACE

At its Seventh Session in October 1973, the FAO Advisory Committee on Marine Resources Research (ACMRR) agreed to establish, jointly with the International Association for Biological Oceanography (IABO), a Working Party on Biological Effects of Pollutants, whose task would be to review and evaluate critically the methods for bioassays and toxicity tests presently used and, specifically, to advise on the practical use of the tests and to identify research programmes required to improve methodology and their application.

Under the cooperative project of the United Nations Environment Programme (UNEP), entitled "Effects of Pollutants on Aquatic Resources and Scientific Basis for Monitoring", the Working Party held two sessions and produced as its end product the Manual of Methods in Aquatic Environment Research, Part 4, Bases for Selecting Biological Tests to Evaluate Marine Pollution (FAO Fish.Tech.Pap.(164), 1977). Furthermore, the Working Party recommended that a manual describing basic toxicity tests be prepared to assist laboratories in developing countries. Based on that recommendation, the present manual has been prepared by G.S. Ward and P.R. Parrish, EG&G Bionomics Marine Research Laboratory, Pensacola, FL, U.S.A.

The views expressed in the manual are those of the author and do not necessarily represent the views of either FAO or UNEP.

SUMMARY

With increasing awareness of aquatic pollution and subsequent moves towards its control, toxicity tests can assist in providing a rationale for such control. Their scope is the assessment of the lethal (acute) or sublethal effects of single or complex solutions of pollutants on tests organisms. Being meant as an elementary guide, the manual lays emphasis on acute static and flow-through toxicity tests. It gives a detailed outline of some basic-type laboratory tests that may be selected in response to the special needs they are aimed to meet, including general instructions for treatment of various kinds of pollutants, i.e. 'natural' effluents. The greatest part is dedicated to the description of the preparation and the test procedures proper for tests on algae, macroinvertebrates and fish. Data analysis and interpretation, particularly statistical procedures and calculations, are only shortly summarized since a bulk of easily-accessible literature exists which is partly referenced.

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1. INTRODUCTION

1.1 Objectives of Aquatic Toxicity Tests

In a toxicity test, two or more treatments are used to study the effect of a test material on test organisms which are usually all of the same species. Although toxicity tests with aquatic organisms can be conducted by applying the test material directly to the test organisms (such as by injection or in food), most tests, especially acute tests, are conducted by exposing the test organisms to test solutions which contain various concentrations of the test material. One or more control treatments are used to provide a measure of the acceptability of the test by giving some indication of the healthiness of the test organisms and the suitability of the dilution water, test conditions, handling procedures, etc. A control treatment is an exposure of the test organisms to dilution water with no test material added. The other treatments are exposures of the test organisms to dilution water with test material added. The test material can be one or more pure chemicals or a complex mixture such as a formulation or an effluent. Sometimes the test solutions are not true solutions because they contain undissolved test material. Test solutions are often prepared by dissolving a test material in a solvent, preferably water, to form a suitable stock solution, and then by adding a portion of the stock solution to dilution water. The objective of a toxicity test is to define the concentrations at which a test material is capable of producing some selected response, usually deleterious, in a population under controlled conditions of exposure. The appropriate way to do this is by use of the quantal response (that is, by having only two experimental alternatives - dead or alive, all or none) from which the relation between concentration and percentage effect can be defined. Quantal data allows statistical definition of the position and slope of the concentration-mortality curve for each selected period of exposure and allows the error at any time to be expressed in the units in which the information is required, that is, concentration (Fig. 1). This information permits the construction of a "toxicity curve" with the confidence limits for each observation shown so that the validity and limits of a subjectively fitted curve can be assessed (Fig. 2).

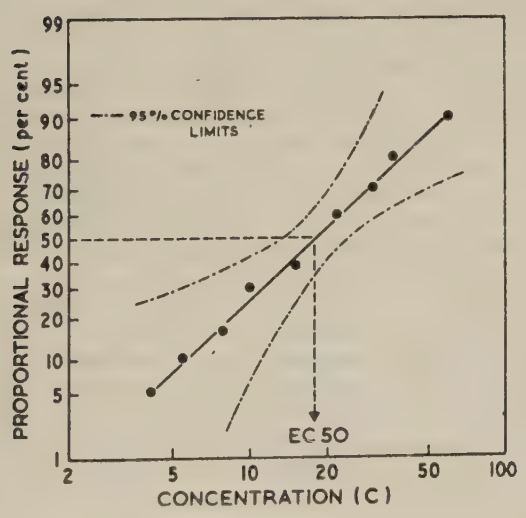

Figure 1. Quantal response of nine test populations after time T_1 (distribution of percentage responded in each test population under nine conditions of exposure T_1C_1, T_1C_2--- T_1C_9)
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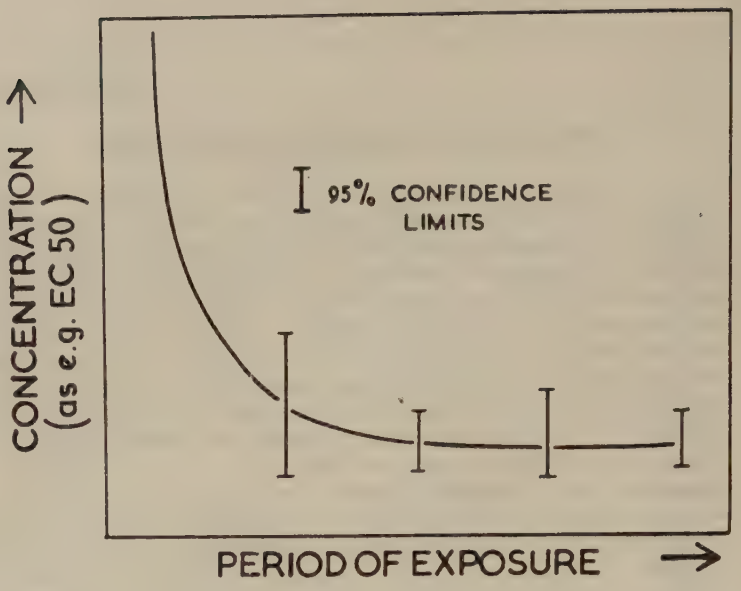

Figure 2. Toxicity curve using quantal data
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In their simplest application, acute toxicity tests are time-dependent. That is, the length of exposure is pre-determined, usually 48 to 96 hours. These tests are a useful tool for routine screening of potentially hazardous test materials and in many cases the effects of test material occur rapidly and are well defined in these time periods. However, because some test materials will not reach a threshold (the point in time where no significant increases in mortality or effect occur) within a 96-hour period, the "toxicity curve" will not be completed. Thus, a time-independent test is a better test for determination of the acute toxicity of a test material than is a time-dependent test. The advantages of the time-independent test are (a) it allows the toxicities of different test materials to be compared easily and meaningfully, and (b) the threshold value serves advantageously as a basic measuring unit for predicting joint toxicity of two or more test materials and for describing effects which are non-lethal.

To exemplify the advantages of the time-indepdent tests, as they relate to comparisons of different test materials or species, Brown (1973) provided the following situations. In the first situation, the average fish of the population exposed to test material E is more sensitive than the average fish exposed to test material D after time T_1. After time T_2, however, the reverse is true (Fig. 3). In this case, the slopes of the two test materials were so different that interpretation of the relative toxicity of the test materials based on a 96-hour exposure may well be reversed with increasing duration of exposure. The same comment applies to comparisons of sensitivities of different species of fish after arbitrary periods of exposure. For example, for two species F and G it may be that the EC50 (the concentration of test material estimated to cause an effect to 50% of the test organisms at specified times) of G is greater than that of F (that is, an average fish of species G is less sensitive than an average fish of species F) (Fig. 4). However, the slopes of the concentration-mortality curves may differ so much that the EC90 for species F is greater than that for species G. Consequently, instead of species F (the apparently more sensitive species on the basis of the EC50) being adversely affected by a certain increase in the concentration of the test material, species G could suffer this fate instead. A similar situation is shown for species F and H, except that in this case both species have the same EC50.

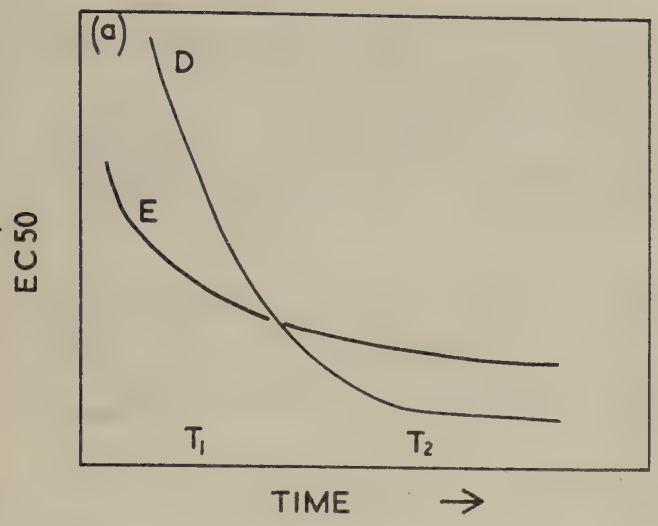

Figure 3. Comparison of toxicities of two test materials (From Brown, 1973, with kind permission of Ann Arbor Science Publishers Inc., Ann Arbor, Mich. © 1973)

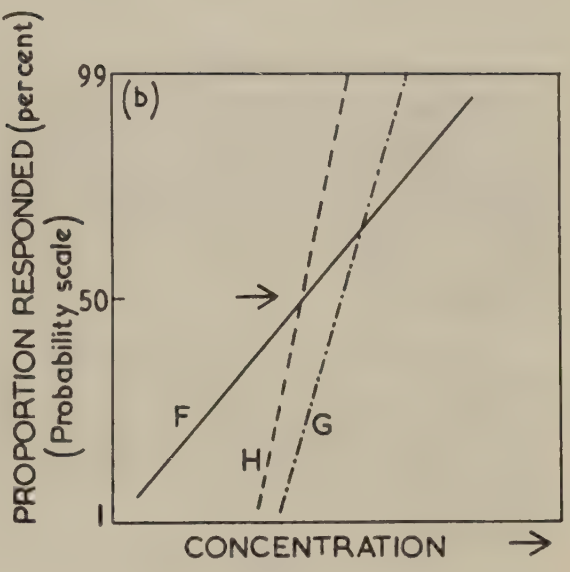

Figure 4. Comparisons of species sensitivities to a test material (From Brown, 1973, with kind permission of Ann Arbor Science Publishers Inc., Ann Arbor, Mich. © 1973)

A 96-hour toxicity test with a unicellular alga can be conducted similarly to the general tests described here and provide an approximation of the toxicity of a test material and comparative toxicity. However, the time-independent approach is not a viable alternative for algal testing because algae are a constantly growing population where even a short-term toxicity test represents a long-term exposure of the culture to a test material because of the rapid growth of the algal culture. This rapid growth also means that algal populations can recover rapidly from a short-term toxic exposure if the population is not killed. Thus, a better approach is the determination of the algistatic concentration in a 14-day test (5 days of exposure and 9 days of recovery). The algistatic concentration represents that concentration of a test material that causes no net change in the test algae population after chronic exposure. Inclusion of the recovery period following the exposure to the test material makes it possible to evaluate the extent of cell damage. The algistatic concentration then can be interpreted as that test material concentration that causes temporary inhibitory effects but does not cause permanent or irreversible damage to the culture.

1.2 Reasons for Acute Toxicity Tests

As was stated previously, the objective in conducting most acute toxicity tests is to define the concentrations at which a test material is capable of producing some selected deleterious response in a population of test organisms. However, acute toxicity tests also serve several other purposes. These include predicting joint toxicity of mixtures of two or more test materials, examination of the effects of modifying factors on the toxicity of a test material (so-called "mitigating" effects), examination of non-lethal effects such as behavioural changes, and as indicators of the need for further testing, including bioconcentration and life-cycle tests.

1.2.1 Joint toxicity

A simple approach for determining how to classify the interaction of two test materials is to expose test organisms to half the concentration of test material A necessary to produce a given response and half the concentration of test material B necessary for the same response. If this combination just causes the response, the actions of A and B are exactly additive. If it causes more than the given response of A or B alone, then they are more-than-additive. If it does not cause the response, then the test materials are either less-than-additive, show no interaction, or are antagonistic, and further experimentation is indicated.

The combination of two toxicants is diagrammed in Figure 5. The axes represent concentrations. The concentration of 1.0 unit of test material A produces death in the absence of test material B and 1.0 unit of B will do the same in the absence of A. If the response is produced by combinations of the two test materials represented by points inside the square, the test materials are enhancing the toxicity of one another. This is called joint action and may be further divided into three special cases as follows:

(1) If the response is just produced by combinations represented by points on the diagonal straight line, the effects are said to be additive.

(2) If the response is produced by combinations falling in the lower left triangle, the effect is more-than-additive.

(3) If the response is in the upper right triangle, the test materials are still working together in joint action but are less-than-additive.

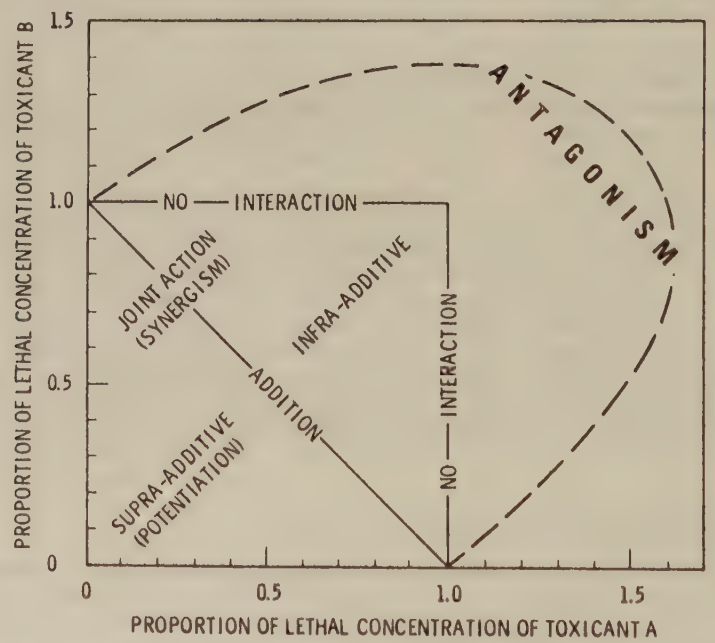

Figure 5. Diagram showing terms used to describe the combined effects of two pollutants (After Gaddum, 1948, with kind permission of Oxford University Press, London. © 1978)

Those combinations falling exactly on the upper and right boundaries of the square show no interaction between the test materials. For example, if 1.0 unit of A is required to just produce the response, no matter what concentration of B is present (as long as it is 1.0 unit), then A is causing the response and B is neither helping nor hindering. If more than 1.0 unit of A is required to produce the effect because of the presence of B, this is antagonism with B antagonizing the effect of A. Any combination of concentrations which would fall outside the square would represent antagonism which is loosely represented by the broken curved line.

This system for predicting joint toxicity is simple and is based on incipient or time-independent LC50s (the concentration of test material estimated to be lethal to 50% of the test organisms when acute lethality has ceased) of the test materials, or reasonable approximations such as 96-hour LC50s (the concentration of test material estimated to be lethal to 50% of the test organisms after 96 hours of exposure). The strength of a given test material (measured in any suitable chemical units) is expressed as a fraction or proportion of its lethal threshold concentration (measured in the same units) when 1.0 toxic unit equals the incipient LC50. Hence, if this number is greater than 1.0 toxic unit more than half of a group of test organisms will be killed by exposure to this test material. If the number is less than 1.0 toxic unit, then half of the test organisms will not be killed. Thus the strength of any test material may be calculated as follows:

$$\text{Toxic units} = \frac{\text{actual concentration in solution}}{\text{lethal threshold concentration (LC50)}}$$

For a mixture, the number of toxic units may be calculated for each of the component test materials. Since the strength of all are expressed in the same units, they may be added together (EIFAC, 1980).

1.2.2 Modifying factors

The toxicity of a given test material may be modified by physical and chemical properties of the dilution water such as temperature, pH, hardness, bicarbonate alkalinity, total dissolved solids, salinity and dissolved oxygen. The test material can be tested through a range of the selected dilution water properties. If the response falls below the lower confidence limits of the established LC50, then less-than-additive interaction occurs. If the response is within the confidence limits, then no interaction occurs. And if the response is beyond the upper confidence limit, then more-than-additive interaction occurs.

1.2.3 Non-lethal effects

In some acute tests, lethality either cannot be measured easily because of the species being tested or lethality occurs at concentrations of test material above an easily quantitated non-lethal effect concentration. Examples of effects used as non-lethal criteria are immobilization (which is defined as the lack of movement except for minor activity of appendages) with daphnids and midge larvae, and loss of equilibrium with crabs, crayfish and shrimp. Other effects can be used for determining an EC50, but the effect and its definition must always be reported. Behavioural changes in such areas as feeding behaviour, sexual behaviour, parental care, social behaviour, defensive behaviour, phototactic responses and diel or tidal rhythms can also be examined in acute toxicity tests but care should be taken to provide the test animals with chemical and biological surroundings similar to what they would encounter in the field.

1.2.4 Indicators of further testing

The basic screening test not only provides an approximation of the toxicity but can also indicate the need for further testing. Bio-concentration or chronic tests might be warranted if the "toxicity curve" drawn from a time-independent test shows continually increasing mortality or non-lethal effects with time because the mortality or effects may reflect cumulative toxicity.

1.3 Types of Acute Tests

1.3.1 Static test

In the static acute test, test solutions and test organisms are placed in test chambers and kept there for the duration of the test. Static acute tests are usually no longer than 96 hours because of problems which arise with tests of longer duration, including the decrease of dissolved oxygen concentrations below acceptability (approximately 60% of saturation, although this varies with the species tested). Other problems may be that metabolic products increase above desired concentrations or that the test material concentrations are lowered significantly because of uptake by the test organisms or degradation within the test chambers.

1.3.2 Renewal test

The renewal acute test is similar to the static acute test except the test organisms are periodically exposed to fresh test solution of the same composition, usually once every 24 hours, either by transferring the test organisms from one test chamber to another or by replacing the test solution. In most cases, the renewal test eliminates problems with dissolved oxygen concentrations, metabolic products, and the lowering of the test material concentration, thus permitting tests of longer duration. However, the renewal test has the disadvantages of possibly stressing the test organisms by increased handling and increased costs of testing because of additional effort required.

1.3.3 Flow-through test

In flow-through acute tests, test solutions flow into and out of the test chambers on a once-through basis for the duration of the test. Test solutions are prepared continuously or every few minutes in a test material delivery system. The flow-through test is the preferred acute toxicity test technique and is almost mandatory for tests which last longer than 96 hours because they provide for continual addition of test solution to the test chambers, maintenance of the dissolved oxygen and test material concentrations and pH at desired concentrations, and removal of degradation and metabolic products.

There are basically two types of flow-through acute tests: the time-dependent and the time-independent.

1.3.3.1 Time-dependent test

The time-depedent test is just that, a test in which the time of termination is predetermined. Commonly, the 96-hour period has been used. The fault with this method is that the shape of the toxicity curve may not be clearly established during the 96-hour period.

1.3.3.2 Time-independent test

The time-independent test does not have a predetermined temporal endpoint. This test is allowed to continue until mortality (or whatever effect criterion is being used) has ceased and the toxicity curve has revealed a threshold. Usually this is 7 to 10 days, although it may be longer. For some test materials, a threshold may not be reached within 21 days, in which case the test might be stopped for practical or economic reasons. If no threshold is apparent it should be clearly reported and care should be taken in choosing concentrations in a test of greater duration (bioaccumulation or life cycle), if one is to be conducted.

2. SAMPLE TREATMENT

2.1 Sample Collection

Any material to be tested in order to evaluate its potential toxicity should be representative of the material as it is used or enters the aquatic environment. The test material sample may be collected by a single "grab" sample if the quality of the material remains relatively similar. In the case of effluents, continuous sampling is preferable. Composite-grab sampling is recommended for static tests where sample quality is relatively stable. For static effluent testing, a composite sample consisting of grab samples collected at each of four consecutive 6-hour periods is taken. Collect samples of effluents that are not constant in composition at different times; do not combine them because knowledge of the maximum, rather than average, toxicity of a variable effluent is more important. It is better to run a flow-through toxicity test with an effluent of variable quality. If a flow-through test is to be run and continuous sampling is impractical, then a composite sample should be collected and transported to the effluent-delivery system every six hours. Each new sample should be used only for the 6 hours between sample delivery.

Composite sampling is achieved by directing a continuous stream of effluent into the sampling container or by taking aliquots (at least 4 per hour) and delivering them to the sample storage container. Either automatic or manual collection of the sample is acceptable. Effluent samples should completely fill the sample container and be sealed tightly.

2.2 Transportation and Storage

Test materials that are considered stable may be transported in appropriate containers with no problems associated with reasonable delivery time. However, effluents should be transported at a low temperature and delivered to the testing facility within 24 hours after collection. Test materials other than effluents should be stored in a cool, dark place. Volatile test materials should be tightly sealed and kept refrigerated, but allowed to come to room temperature before testing. Effluents, especially those containing organic matter subject to bacterial decomposition, should be refrigerated at $>0<4^{o}C$. Effluents should not be frozen.

2.3 Preparation of Test Material Solution

Solutions of toxicants should be prepared in advance so that they may be added immediately to the dilution water for static tests in the required amounts to obtain the different test concentrations. All solutions required for each series of toxicity tests should be prepared from the same sample of material. Any undissolved material should be uniformly dispersed by shaking before a sample is withdrawn and added to dilution water in the test containers. Tests should be conducted with the test material added directly to the dilution water without the use of any solvent/carrier, except water, if possible. If a solvent/carrier is necessary, the amount used should be kept to a minimum. The organic solvents triethylene glycol (TEG) and dimethylformamide (DMF) are the preferred solvent/carriers because of their low toxicity to aquatic organisms, low volatility, and their ability to dissolve many organic test materials. However, other solvent/carriers may be used including acetone, methanol and ethanol. Whenever a solvent/carrier other than water is used, it is recommended that the solvent/carrier concentration in any test solution not exceed 0.5 ml/l. There may be cases in which this limit cannot be met and in those cases higher concentrations are permitted, as long as an appropriate solvent/carrier control is maintained and no signs of toxicity to the test organisms are exhibited in the solvent/carrier control.

Effluent tests should be conducted with effluent which has not been aerated or altered in any way. Undissolved materials must be dispersed by gentle agitation immediately prior to removal of a subsample. In flow-through tests, undissolved material may be kept in suspension by use of a stirring device.

The effluent can be used directly as a stock solution or a stock solution can be prepared by diluting the effluent with filtered sea water or fresh water to the desired concentration and volume for mixing with the dilution water. Stock solutions or test solutions should be prepared on a volume:volume ratio of dilution water and liquid effluent so that the percentage of effluent in each of the test solutions can be calculated. If the effluent is a solid waste, dilution should be on a weight:volume basis. If a volume:volume basis is used and it is desired to express it as weight per volume concentration, a correction should be made for specific gravity.

3. ALGAL TOXICITY TESTS

Algal materials and methods presented are based on those in "Proposed standard practice for conducting toxicity tests with freshwater and saltwater algae" (ASTM, 1980), and those of Miller et al. (1978) and Payne and Hall (1979).

3.1 Synthetic Algal Nutrient Medium

All chemicals used to prepare algal growth medium should be reagent grade or better. Reagent water for freshwater algal tests should conform to the following specifications:

Total matter, max, (mg/l)	1.0
Electrical conductivity, max microohms (μmho)/cm at 298 Kelvin (K) (= 25°C)	1.0
Electrical resistivity, min, MΩ/cm at 298K (= 25°C)	1.0
pH at 298K (= 25°C)	6.2 to 7.5
Minimum colour retention time of potassium permanganate, minutes	10
Maximum soluble silica	10 μg/l

The reagent water shall be prepared by distillation, ion exchange, reverse osmosis or a combination thereof, followed by polishing with a 0.45-μm pore size membrane filter. Specifications for reagent water are based on Type III reagent water as designated by the American Society for Testing and Materials (ASTM, D1193, 1978 Annual Book of ASTM Standards, Part 31) (ASTM, 1978) or equivalent. References to water shall be understood to mean reagent water and references to sea water shall be understood to mean natural filtered sea water.

3.1.1 Freshwater maintenance and test medium preparation

Freshwater algae are cultured and tested in the same medium, except Na_2EDTA is omitted from test medium when metals are tested.

Prior to preparing test medium, macronutrient stock solutions must be prepared (each solution is prepared separately) as follows:

(1) Dissolve 25.5 g $NaNO_3$ in 1 l water

(2) Dissolve 12.2 g $MgCl_2 \cdot 6H_2O$ in 1 l water

(3) Dissolve 14.7 g $MgSO_4 \cdot 7H_2O$ in 1 l water

(4) Dissolve 4.41 g $CaCl_2 \cdot 2H_2O$ in 1 l water

(5) Dissolve 15.0 g $NaHCO_3$ in 1 l water

(6) Dissolve 1.044 g K_2HPO_4 in 1 l water

Prepare micronutrient stock solutions as follows:

(1) Dissolve 0.78 g $CoCl_2$ in 1 l water

(2) Dissolve 0.9 g $CuCl_2$ in 100 ml water
Dilute 1 ml of this solution to 1 l for working stock solution

(3) To 1 l of water add 0.1855 g H_3BO_3, 0.2643 g $MnCl_2$, 0.0327 g $ZnCl_2$, 0.0073 g $Na_2MoO_4H_2O$, 0.0960 g $FeCl_3$, 0.300 g Na_3EDTA and 1.0 ml of micronutrient numbers (1) and (2)

The maintenance and test medium is prepared by adding 1 ml of each macronutrient solution and 1 ml of the micronutrient stock solution number (3) to 1 l of water.

Maintenance medium is sterilized by autoclaving for 30 minutes at 121°C and 1.1 kg/cm^2. Test medium is sterilized by vacuum filtration through 0.45-µm pore size filters and equilibrated for 24-48 hours.

3.1.2 Marine algal maintenance medium

This medium is used exclusively for maintaining stock cultures of marine algae. Preparation of metal solutions are as follows (each solution is prepared separately) and solutions must be stored in a freezer.

(1) Dissolve 4.8 g $FeCl_2 \cdot 6H_2O$ in 100 ml water

(2) Dissolve 14.4 g $MnCl_2 \cdot 4H_2O$ in 100 ml water

(3) Dissolve 4.5 g $ZnSO_4 \cdot 7H_2O$ in 100 ml water

(4) Dissolve 0.0157 g $CuSO_4 \cdot 5H_2O$ in 100 ml water

(5) Dissolve 0.0404 g $CoCl_2 \cdot 6H_2O$ in 100 ml water

The metal stock solution is then prepared by adding 1 ml of each metal mix solution, 1 g Na_2EDTA, and 1.14 g H_3BO_3 to 1 l of water.

Preparation of vitamin solutions are as follows (each solution is prepared separately).

(1) Dissolve 0.1 g biotin in 100 ml of 95% ethanol

(2) Dissolve 1.0 g vitamin B_{12} in 100 ml of water

The vitamin stock solution is then prepared by adding 1 ml of biotin vitamin solution, 1 ml of vitamin B_{12} stock solution, and 5 g of thamine HCl to 100 ml of water.

Preparation of the salt mix consists of adding 3 g of K_3PO_4, 50 g $NaNO_3$, and 20 g $NaSiO_3 \cdot 9H_2O$ to 1 l of water.

The maintenance medium is prepared by dissolving 30 g of a commercial or prepared synthetic seawater mix in 1 l of water (Table I). After filtering (0.45-µm pore size filter), supplement the solution with 30 ml of metal stock solution, 1 ml of vitamin stock solution and 2 ml of the salt mix. The maintenance medium is then sterilized by autoclaving at 121°C for 30 minutes at a pressure of 1.1 kg/cm^2.

3.1.3 Marine algal test medium

Stock cultures of marine algae are acclimated and tested in this medium. Whenever possible, natural filtered sea water should be used as the test medium after supplementing with nutrients; however, the maintenance medium described previously is also acceptable.

Table I

Synthetic seawater[1/] formulation for algal assays
(After Kester et al., 1967, with kind permission of
American Society of Limnology and Oceanography. © 1967)

Compound	Concentration/litre
NaCl	24.00 g
Na_2SO_4	4.00 g
H_3BO_3	0.03 g
$CaCl_2 \cdot 2H_2O$	1.47 g
$MgCl_2 \cdot 6H_2O$	10.78 g
$Na_2SiO_3 \cdot 9H_2O$[2/]	0.03 g
KCl	0.70 g
$NaHCO_3$	0.20 g

The macronutrient stock solution for the test medium is prepared by adding 4.42 g $NaNO_3$ and 0.87 g $K_2HPO_4 \cdot H_2O$ to 1 l of water.

Vitamin solutions are prepared as follows and should be stored in a freezer.

(1) Dissolve 500 mg biotin in 1 l water

(2) Dissolve 500 mg vitamin B_{12} in 1 l water

The vitamin stock solution is prepared by adding 100 mg thiamine HCl, 1 ml biotin solution, and 1 ml vitamin B_{12} solution to 1 l of water.

The trace metal solutions are prepared as follows:

(1) Dissolve 21.3 mg $CuCl_2 \cdot 5H_2O$ in 100 ml water

(2) Dissolve 20.8 mg $ZnCl_2 \cdot 5H_2O$ in 100 ml water

(3) Dissolve 40.0 mg $CoCl_2 \cdot 6H_2O$ in 100 ml water

(4) Dissolve 35.7 mg $MnCl_2 \cdot 4H_2O$ in 100 ml water

(5) Dissolve 11.4 mg $Na_2MoO_4 \cdot H_2O$ in 100 ml water

The trace metal stock solution is then prepared by adding 1 ml of each trace metal solution and 0.54 g $FeCl_2 \cdot 6H_2O$ to 1 l of water.

The test medium is prepared by adding 10 ml of macronutrient stock solution, 1 ml of vitamin stock solution, 1 ml of trace metal stock solution, and 0.5 g Tris buffer. The test medium is sterilized by vacuum filtration through 0.45-µm pore size filters and equilibrated for 24-48 hours.

[1/] Completed medium must be stripped of contaminant trace metals before use in bioassays (Davey et al., 1970). In order to reduce contamination by trace metals, this medium is treated with Chelex-100 (Davey et al., 1970). Salinity should be adjusted to 30 per mille prior to stripping. Readjusting pH to 7.8 -8.2 after stripping.

[2/] Prepare a stock solution in deionized water and adjust to pH 7.8. Add slowly with constant mixing to completed medium at 7.8-8.2 pH.

3.2 Test Algae

The recommended alga for freshwater testing is *Selenastrum capricornutum* and *Skeletonema costatum* for saltwater testing.

3.2.1 Source of test algae

Algal cultures may be secured from the National Eutrophication Research Program, Pacific Northwest Environmental Research Laboratory, National Environmental Research Center, Environmental Protection Agency, 200 S.W. 35th Street, Corvallis, Oregon 97330, U.S.A., and the Department of Botany, University of Texas at Austin, Texas 78712, U.S.A.

3.2.2 Maintenance of stock algal cultures

Upon receipt of an algal culture, a portion should be aseptically transferred to algal maintenance medium. The volume transferred is not critical; however, be sure enough cells are included to overcome significant growth lag (i.e. 1.0-ml culture added to 50 ml in a 125-ml flask). Weekly aseptic routine stock transfer is recommended to maintain a continuous supply of "healthy cells" for experimental work. Extreme care should be exercised to avoid contamination of stock cultures. To retain an unialgal culture over a long period of time it is advantageous to prepare a semi-solid medium containing 1% agar. This semi-solid medium is placed in sterile Petri dishes. A portion of a liquid algal culture is streaked onto the agar medium and incubated under the following conditions:

Temperature:	$24 \pm 2^{\circ}C$ for freshwater algae $20 \pm 2^{\circ}C$ for marine algae
Illumination:	Continuous "cool-white" fluorescent lighting at 4 300 lux for freshwater algae and 2 000 lux for marine algae
Gas exchange:	Free exchange through foam plugs with continuous shaking at 100 revolutions per minute (rpm)
Salinity:	30 per mille (‰) for marine algae

3.2.3 Preparation of *Selenastrum capricornutum* inoculum for testing

Each test flask and control are to be inoculated with a final algal concentration of approximately 1.0×10^4 cells per ml. After determining the initial algal cell concentration in a 7-8 day old stock culture, determine the volume of stock culture required to prepare inoculum using the following equation.

$$\frac{\text{(A) Final volume of inoculum (ml)} \times \text{(B) Final concentration of inoculum in test flask (cells/ml)} \times \text{(C) Volume of solution in test flasks (ml)}}{\text{(D) Initial algal cell counts}} = Q$$

Q = Volume (ml) of stock culture required to prepare inoculum

EXAMPLE: 180 flasks containing 100 ml of solution (C) are required for the test. Each flask is to be inoculated with 10 000 cells/ml (B) final concentration. 200 ml of suspended algal cells (A) should be prepared to ensure an adequate amount of inoculum. The product of (A), (B) and (C) is divided by the algal cell count (D), 2 000 000 cells. The resulting quotient (Q) indicates the volume (ml) of the stock culture (D) to be used to prepare the 200 ml of inoculum. This inoculum should contain a final concentration of $1\ 000\ 000 \pm 1\%$ cells/ml, one ml of which when added to 100 ml of test solution results in a final algal cell concentration in the test flask of 10 000 cells/ml.

3.2.4 Preparation of *Skeletonema costatum* inoculum for testing

Transfer 1.0 ml of *Skeletonema costatum* stock culture from the marine algal maintenance medium to flasks containing marine algal test medium. After 7-10 days of incubation under the conditions described in 3.2.2, again transfer 1.0 ml of the *S. costatum* suspension to flasks containing fresh marine algal test medium. Incubate for 7-10 days. The algal cells will be ready for use after the second incubation. Each test flask and control are to be inoculated with a final algal concentration of approximately 1.0×10^4 cells per ml. After determining the initial algal cell concentration, determine the volume of stock culture required to prepare the inoculum using the equation previously provided in 3.2.3.

3.3 Equipment

This standard practice suggests 50 ml of test medium in 125-ml flasks. When the objectives of the test require larger volumes of test medium, the test medium and flask size can be increased to meet the needs of the particular study. Volumes of test medium of 125 ml, 250 ml and 500 ml can be added to 250 ml, 500 ml and 1 000 ml flasks, respectively. Equipment needed for performance of the tests include the following:

1. Centrifuge
2. Centrifuge tubes made of glass or polycarbonate with screw-cap lids
3. Environmental chamber with temperature and lighting controls
4. Rotary shaker(s) with variable speed control to 100-120 rpm
5. Erlenmeyer flasks made of borosilicate glass
6. Foam rubber plugs or stainless steel caps for flask stoppers
7. Pipettes, Eppendorf or equivalent
8. Filtration apparatus
9. Membrane filters, 0.45-µm pore size
10. Analytical balance
11. Autoclave
12. pH meter
13. Calibrated light meter
14. Microscope capable to 100-400X magnification
15. Haemacytometer counting chamber and ocular micrometer
16. Electronic particle counter, mean cell volume accessory, aperture tube with 100-µm opening
17. Fluorometer equipped to measure chlorophyll a

Determination of cell counts can be performed using either a haemacytometer and microscope or an electronic particle counter. All other equipment is necessary for all tests.

3.4 Test Methods

3.4.1 Range-finding test

A range-finding (preliminary) test is conducted to determine the concentrations of test material to be used in the definitive test. To set up a preliminary test, add 50 ml of the appropriate growth medium into 125-ml flasks. Each concentration should be established in duplicate. Add the test material to the flasks in concentrations that range at least four orders of magnitude (e.g. 0.1, 1.0, 10 and 100 mg/l (= ppm)). Inoculate each test flask

with a final algal concentration of 1 x 10^4 cells per ml. Incubate the flasks under the conditions described earlier in 3.2.2. Duration of incubation is 4 to 5 days depending on the type of definitive test to be conducted. Determine the growth in each test flask compared to that which occurred in the algal test medium or solvent control. Always compare the results of the test material flasks to the solvent control flasks when a solvent is used to dissolve the test material.

3.4.2 Definitive test

The definitive test is conducted by using concentrations of test material selected from the results of the range-finding test. Place 50 ml of appropriate test medium into 125-ml flasks. Each concentration should be established in triplicate for the definitive test. A minimum of five concentrations of the test material must be used. Each concentration of test material should be 50% of the next concentration. Inoculate each test and control flask with a final algal concentration of approximately 1 x 10^4 cells per ml. Incubate the flasks under the conditions described in 3.2.2. For a 4-day test, *in vivo* chlorophyll *a* is measured at 24-hour intervals. Cell counts are made after appropriate length of exposure. For a 14-day test, the algae are exposed to the test material for 5 days and are then transferred to fresh test medium for a period of 9 additional days. Determination of *in vivo* chlorophyll *a* should be made on days 3, 5, 7, 9, 11 and 14. Cell counts are made on days 5 and 14. After five days of exposure to the test material, cells and test medium from the triplicate flasks of each treatment are combined and centrifuged at 1 000X centrifugal force (G). The cells are then resuspended in test material-free medium, centrifuged and resuspended in 50 ml of appropriate test medium for a 9-day recovery period.

3.5 Statistical Analyses of Results

For 96-hour tests, the 24-, 48-, 72- and 96-hour EC50s and 95% confidence limits are calculated. After each test concentration is converted to a logarithm and the corresponding percentage increase or decrease of *in vivo* chlorophyll *a* or cell numbers is converted to a probit, the EC50s are calculated by linear regression (Finney, 1971). To determine if growth in the solvent control (where applicable) differed from that of the growth medium control, data are analysed by "Student's" t-test (Steel and Torrie, 1960). Differences are considered significant at the 95% confidence level ($P \leq 0.05$).

For 14-day tests, a graphically interpolated minimum algistatic concentration (MAC-5) is determined. The MAC-5 is the minimum concentration resulting in an algistatic response after 5 days of exposure, based on cell numbers (Payne and Hall, 1979). After the 9-day recovery period in test substance-free medium, growth constants are calculated according to the relationship

$$k = \frac{\log 2\ (N_1/N_2)}{t}$$

where k is the growth constant expressed as generation or doubling time per day, and N_1 and N_2 are cell numbers (at the end and beginning of the recovery period, respective) and t is the period of exposure in days. To determine whether growth constants derived for algal cultures previously exposed to the test substance were significantly different from growth constants derived for the control(s), data are analysed by "Student's" t-test. Differences are considered significant at the 95% confidence level.

3.6 Standard Toxicant

The use of a standard toxicant is recommended for all algal tests and should be tested with the same inoculum and test medium being used to test the material of interest. By comparing the results of each standard toxicant test (conducted under the same test conditions as the test material), the investigator will have assurance that technical aspects of the test were properly performed. Dodecyl sodium sulfate (DSS) has proven useful in many laboratories as the standard toxicant. This surfactant produces a well-defined response between 1 to 3 ml/l and is soluble and stable in algal test medium.

3.7 Data Interpretation

This method is intended to provide information concerning the effects of selected test materials on representative freshwater and saltwater algae. It must be noted that a test material may stimulate, as well as inhibit, algal growth or can cause death of the algal cells. The 14-day test, in addition to allowing for determination of EC50s, also allows for quantification of toxic responses ranging from algistatic to algicidal. The primary toxic effect sought in this method is the algistatic response. The algistatic concentration of a test material is a concentration that prevents the test alga from further growth. However, the algae will recover and grow when subcultured into fresh growth medium. The algistatic concentration then can be interpreted as that test material concentration that causes temporary inhibitory effects but does not cause permanent or irreversible damage to the culture.

4. MACROINVERTEBRATES AND FISH TOXICITY TESTS

Materials and methods presented for macroinvertebrate and fish toxicity tests are based on those of Peltier (1978), Sprague (1969, 1970) and EPA (1975).

4.1 Dilution Water

An adequate supply of dilution water that is acceptable to the test organisms and to the purpose of the test must be available. The minimal criterion for an acceptable dilution water is that healthy test organisms will survive in it for the duration of the acclimation and testing without showing signs of stress, such as discolouration or unusual behaviour. Dilution water should be aerated prior to the introduction of the test material. Adequate aeration will bring the pH and concentration of dissolved oxygen and other gases into equilibrium with air and minimize oxygen demand and the concentration of volatiles. The concentration of dissolved oxygen in the dilution water should be greater than 90% of the saturation. Dilution water which may contain undesirable micro-organisms should be passed through a properly maintained ultraviolet sterilizer equipped with an intensity meter.

For effluent tests, whenever possible, the dilution water should be a representative sample of the receiving water obtained as close to the point of discharge as possible, but upstream of or outside of the zone of influence of the effluent. If an acceptable dilution water cannot be obtained from the receiving water or aerated receiving water, some other uncontaminated, well-aerated surface, ground, or reconstituted water with hardness, alkalinity and specific conductance within 25% and pH within 0.2 unit of those of the receiving water at the time of the test may be used.

The dilution water should be uncontaminated and of constant quality and should meet the following specifications:

Suspended solids	< 20 mg/l
Total organic carbon (TOC)	< 10 mg/l
Un-ionized ammonia	< 20 µg/l
Residual chlorine	< 3 µg/l
Total organophosphorus pesticides	< 50 ng/l
Total organochlorine pesticides plus Polychlorinated biphenyls (PCBs)	< 50 ng/l

4.1.1 Fresh water

Freshwater dilution waters should be obtained from an uncontaminated well or spring if possible; only as a last resort should dechlorinated water be used. Whenever possible, a dilution water with a hardness of 40-200 mg/l as $CaCO_3$ should be used for acute tests.

A freshwater dilution water is considered to be of a constant quality if the monthly ranges of the hardness, alkalinity and specific conductance are less than 10% of their respective averages and if the monthly range of pH is less than 0.4 unit.

4.1.2 Salt water

A brackish or marine dilution water is considered to be of a constant quality if the weekly range of pH is less than 0.8 unit. Whenever possible, a salinity of 28-34‰ should be used when testing stenohaline species, and a salinity of 15-25‰ should be used for tests with euryhaline species.

4.2 Test Animals

Preferably, when assessing possible pollution effects, test animals should be indigenous to the area of impact. The test animals can be either obtained directly from wild populations in relatively unpolluted areas, purchased from commercial suppliers or cultured in the laboratory. If species are not available from a local area, then representative species from nearby localities offer an alternative source. Animals must not be collected by electroshocking. All animals in a given test should be from the same source.

4.2.1 Field collection and transport

The capture and transport of animals should be done in a manner which minimizes injury and physiological trauma. For example, fishes such as Atlantic silversides (*Menidia menidia*) or any of the herrings and anchovies are easily captured by shore seine. However, rather than beaching the seine, it is far less deleterious to the fish to scoop them with a soft net from the water and into a transport container while they are still swimming within the enclosed net in shallow water. When beam or other trawls must be used, slow tows which are very short in duration are suggested.

Ideally, transport containers, especially for highly motile species, should be circular or elliptical to prevent animals from crowding in corners or damaging themselves by striking the walls (Marine Technology Society, 1974). However, styrofoam containers have proved acceptable, as have other containers cushioned in some fashion such as by the use of plastic bags inside the containers. Maximum density of animals in a container will vary with the requirements of the species. When temporary containment and transporting measures must be sustained for much more than 30 minutes, additional safeguards must be taken to shield the animals from the sunlight and from extreme temperature and dissolved oxygen depletion. Styrofoam coolers provide temporary temperature maintenance. For many slow-moving or sessile animals, large volumes of water are not needed. Items such as wet seaweed may be an ideal transport medium for these animals.

4.2.2 Maintenance, care and handling

When organisms are first brought into the testing facility, they should be quarantined, at least until they appear to be disease-free. To maintain organisms in good condition, crowding should be avoided and the dissolved oxygen concentration should be maintained >60% of saturation. To avoid unnecessary stress, organisms should not be subjected to rapid changes in temperature or water quality. In general, organisms should not be subjected to more than a 5°C gradual change in water temperature in any 24-hour period, and for salt-water organisms no more than a 5‰ change in 24 hours. Organisms should be fed at least once a day and tanks cleaned as necessary. Failure to eat is common to animals recently brought into the laboratory. This initial period of non-feeding may be shortened by social facilitation, i.e. adding animals which are already feeding to the holding tank. (Care should be taken not to indiscriminately mix animals from different lots, however, because of their different histories - age, condition and time in acclimation, for example). When laboratory animals do not begin to feed, or stop feeding once having begun feeding, this may be indicative of an unhealthy condition in the facility. Organisms should be observed carefully during the initial maintenance period for signs of disease, stress, physical damage and mortality. Dead, diseased and abnormal individuals must be discarded.

Organisms should be handled as little as possible. When handling is necessary, it should be accomplished as gently and quickly as possible. Small dipnets are best for handling larger organisms. Such nets are commercially available, or can be made from small-mesh nylon netting, nylon or silk bolting cloth, plankton netting or similar material. Smooth bore glass tubes with fine polished ends and rubber bulbs should be used for transferring smaller organisms such as daphnids and midge larvae.

After collection or transportation, invertebrates should be maintained in holding tanks for at least 2 days. They should be held in uncontaminated dilution water under stable conditions of temperature and water quality in a flow-through system with a flow rate of at least two water volume additions per day or in a recirculating system in which the water flows through a carbon filter.

4.2.3 Acclimation

Test organisms which can be cultured in the laboratory should be reared in the solution water at test temperature. Other organisms can be acclimated (in a flow-through system) simultaneously to the dilution water and test temperature after transferring an appropriate number of similar size individuals from a holding tank to an acclimation tank. Animals should be acclimated to the dilution water by gradually changing the water in the acclimation tank from 100% holding water to 100% dilution water over a period of 2 or more days. They should be acclimated to the test temperature by changing the water temperature at a gradual rate not to exceed 5°C within 24 hours until the test temperature range is reached. Animals should be maintained for at least 2 days at the test temperature before tests are begun with them. Longer acclimation times are generally desirable.

A group of test organisms must not be used for a test if the individuals appear to be diseased or otherwise stressed or if more than 10% die during the 48 hours immediately prior to the beginning of the test. If a group fails to meet these criteria, all individuals must be either discarded or treated and held an additional 4 days.

Organisms should be approximately uniform in size and age. Early life stages of invertebrates and fish which are to be tested, <0.5 g each, and invertebrates with relatively short life cycles (<60 days) must be fed up to the beginning of the test. All organisms >0.5 g should not be fed for 24 hours before the beginning of the test.

4.3 Disease Treatment

Freshwater fish may be chemically treated to cure or prevent diseases by using recommended treatments (Table II), but if they are severely diseased, it is often better to destroy the entire lot. Until acceptable treatments have been proven effective, all other diseased animals should be discarded. Generally, organisms should not be treated during the first 16 hours after they arrive at the facility because they are probably stressed due to collection or transportation and some are treated during transit. Tests should not be begun with treated organisms for at least 4 days after treatment. Tanks and test chambers which may be contaminated with undesirable micro-organisms can be sterilized for 1 hour with an iodophor or with 200 mg of hypochlorite per litre.

4.4 Exposure Apparatus

4.4.1 Static acute tests

Equipment necessary for conducting static acute toxicity tests include an adjustable constant-temperature room or temperature-controlled water bath for the test chambers. The size of the test chambers vary according to the size of the test animals and can be finger bowls, jars, tanks, beakers, crystallizing dishes or any other appropriate container. The test containers should be constructed of materials which do not contain any substances that can be leached or dissolved by the water. In addition, materials and equipment that contact

Table II

Recommended prophylactic and therapeutic treatments for freshwater fish[3/]
(From EPA, 1975, with kind permission of U.S. Environmental Protection Agency)

Disease	Chemical	Concentration (mg/l)	Application
External bacteria	Benzalkonium chloride (Hyamine 1622^{R})	1-2 AI[4/]	30-60 min[5/]
	Nitrofurazone (water mix)	3-5 AI	30-60 min[5/]
	Neomycin sulfate	25	30-60 min[5/]
	Oxytetracycline hydrochloride (water soluble)	25 AI	30-60 min[5/]
Monogenetic trematodes, fungi and external protozoa[6/]	Formalin plus zinc-free malachite green oxalate	25 0.1	1-2 hours[5/]
	Formalin	150-250	30-60 min[5/]
	Potassium permanganate	2-6	30-60 min[5/]
	Sodium chloride	15 000-30 000 2 000- 4 000	5-10 min dip [7/], [5/]
	Dexon^{R} (35% AI)	20	30-60 min[5/]
Parasitic copepods	Trichlorofon (Masoten^{R})	0.25 AI	[8/]

[3/] These recommendations do not imply that these treatments have been cleared or registered for these uses. Appropriate state and federal regulatory agencies should be consulted to determine if the treatment in question can be used and under what conditions the uses are permitted. These treatments should be used only on fish intended for research. They have been found dependable, but efficacy against diseases and toxicity to fish may be altered by temperature or water quality. Researchers are cautioned to test treatments on small lots of fish before making large-scale applications. Prevention of disease if preferred, and newly acquired fish should be treated with the formalin-malachite green combination on three alternate days if possible. However, generally fish should not be treated on the first day they are in the facility. This table is merely an attempt to indicate the order of preference of treatments that have been reported to be effective. Before a treatment is used, additional information should be obtained from sources such as Davis (1953), Hoffman and Meyer (1974), Reichenbach-Klinke and Elkan (1965), Snieszko (1970) and van Duijn (1973.

[4/] AI - active ingredient

[5/] Treatment may be accomplished by (1) transferring the fish to a static treatment tank and back to a holding tank; (2) temporarily stopping the flow in a flow-through system, treating the fish in a static manner, and then resuming the flow to flush out the chemical; or (3) continuously adding a stock solution of the chemical to a flow-through system by means of a metered flow of the technique of Brungs and Mount (1967).

[6/] One treatment is usually sufficient except of "Ich", which must be treated daily or every other day until no sign of the protozoan remains. This may take 4-5 weeks at 5-10°C and 11-13 days at 15-21°C. A temperature of 32°C is lethal to Ich in one week.

[7/] Minimum of 24 hours but may be continued indefinitely.

[8/] Continuous treatment should be employed in static or flow-through systems until no copepods remain, except that treatment should not be continued for over 4 weeks and should not be used above 27°C.

stock solutions of test solutions should be chosen to minimize sorption of toxicants from water. To minimize leaching, dissolution and sorption, containers made of glass, #316 stainless steel and perfluorocarbon plastics should be used whenever possible. Plasticized plastics, rubber, copper, brass or lead must not come in contact with dilution water, stock solutions, effluent samples or test solutions.

4.4.2 Flow-through acute tests

Facilities necessary for conducting flow-through toxicity tests include a constant-temperature room or temperature-controlled dilution water bath for test chambers. There should be a dilution-water tank that may be elevated, so that dilution water can flow by gravity into the test material delivery system. The dilution-water tank should be equipped for temperature control and aeration. Air used for aeration must be free of oil and fumes; filters to remove oil and water are desirable. Construction materials for flow-through toxicity tests are as described previously in 4.4.1. Although many toxicant delivery systems can be used, the proportional diluter (Mount and Brungs, 1967, and Lemke et al., 1978) is probably the best for routine use. The diluter consists of calibrated water cells which receive various volumes of uncontaminated dilution water and calibrated chemical cells which receive various volumes of test solution. The various cells of dilution water siphon down mixing with the various volumes of test solution to produce the test concentrations. Chambers to promote mixing of the test material-containing water and the dilution water are located between the diluter and the test chambers. The flow rates through the test chambers should be $\geq$2 water volume additions per 24 hours.

For effluent tests where high test concentrations might approach 100%, the proportional diluter can be modified by introducing 100% effluent directly into the chemical cells and eliminating the first water and mixing cells.

The calibration of the toxicant delivery system should be checked before each test. This should include determining the flow rate through each test chamber and measuring either the concentration of toxicant in each test chamber or the volume of solution used in each portion of the diluter. The general operation of the diluter should be checked daily during the test.

4.5 Test Conditions

4.5.1 Experimental design

The number of test organisms to be exposed to each treatment is at least 10 for static or flow-through toxicity tests, but they may be divided between two or more test chambers. The use of more organisms and replicate test chambers for each treatment is desirable. A representative subsample of the test organism population should be impartially distributed to the test chambers, either by adding 2 (if there are to be 10 of fewer organisms per chamber) or 4 (if there are to be more than 10 organisms per chamber) test organisms to each chamber, and then adding 2 or 4 more, and repeating the process until each test chamber has the desired number of test organisms in it. For small organisms it is often convenient to assign organisms to other containers first and then gently add them to the test chambers all at once.

Every test requires a control which consists of the same dilution water, conditions, procedures and organisms as are used in the remainder of the test. If a solvent/carrirer or other additive is present in any of the test chambers, a solvent/carrier or additive control is also required. This solvent/carrier or additive control is treated the same as the regular control except that the higher amount of solvent/carrier or additive present in any other test chamber is added to this test chamber. A test is not acceptable if more than 10% of the organisms die in any control in a test determining an LC50 or show the effect in a test determining an EC50.

It is desirable to repeat the test at a later date to obtain information on the reproducibility of the results of the test.

4.5.2 Dissolved oxygen concentration

Test solutions should not be aerated in the test chambers or in the test material delivery system. The dissolved oxygen concentration should remain ≥60% of the saturation during the test. In cases where the dissolved oxygen concentration does fall below 60%, the tester should watch the test organisms carefully for signs of stress. It is often useful to set up an additional test chamber containing the highest concentration of test material (especially an effluent) but without test organisms and to monitor the effect of the test material alone on the dissolved oxygen concentration. Test materials having a known characteristic of rapidly depleting the dissolved oxygen concentration may be tested both aerated and unaerated to determine the toxicity of the test material and that of the test material in combination with low dissolved oxygen concentrations.

4.5.3 Test temperature

The test temperature should be the optimum for the test species and be within the range 7-27°C. The actual test temperature should not deviate from the selected test temperature by more than 1°C during the test. The test temperature should be selected for the purpose of the test.

For effluent flow-through tests, the test temperature should be between the daily low and the daily high temperature of the receiving water measured at the time of the test just outside the zone of influence of the effluent. The test temperature may be constant throughout the test or may fluctuate within the allowable test temperature range with the temperature of the receiving water.

4.5.4 Loading

The grams of organisms per litre of solution (loading) in the test chambers must not be so high that it affects the results of the test. Therefore the loading must be limited to ensure that the concentration of dissolved oxygen and toxicant is not decreased below acceptable concentrations, that the concentration of metabolic products does not increase above acceptable concentrations and that the organisms are not stressed due to crowding. Loading in the test chambers should not exceed 0.5 g/l for static tests or 1 g/l of test solution passing through the test chamber in 24 hours for flow-through tests.

4.5.5 Beginning the test

Static tests are begun by adding test organisms to the test chambers within 1 hour after the toxicant is added to the dilution water. Addition of the test material into the dilution water should be followed by swirling the test solution with a glass or Teflon rod to disperse the test material.

Flow-through tests are begun by placing the test organisms in the test chambers after the test solutions have been flowing through the test chambers long enough so that the test material concentrations are constant.

The test organisms should not be fed while in the test chambers. Minimal feeding may be necessary, however, if (a) cannibalistic animals cannot be separated or restrained, or (b) the test duration constitutes a relatively large portion of the test organism's life span.

A test begins when the test organisms are first exposed to the toxicant. The normally used exposure time is 96 hours; however, certain species such as daphnids may be tested for only 48 hours due to problems associated with longer exposure times.

4.5.6 Biological data

The number of dead or affected organisms in each test chamber must be counted every 24 hours after the beginning of the test. Death is the criterion for effect most often used to study acute toxicity with aquatic organisms. The criteria for death are usually the lack of movement and lack of reaction to gentle prodding. However, because death is not easily determined for some invertebrates or because some other effect occurs at sublethal concentrations which would result in death in the aquatic environment, an EC50 is often determined rather than an LC50. Common effects generally used for determining an EC50 are immobilization and loss of equilibrium. Other effects can be used for determining an EC50, but the effect and its definition must always be reported. General observations on such things as erratic swimming, loss of reflex, discolouration, changes in behaviour, excessive mucous production, hyperventilation, opaque eyes, curved spine, haemorrhaging, moulting and cannibalism, should be reported.

The weights and sizes of test organisms should be determined by measuring representative organisms before the test or the control organisms after the test. Organisms that are to be used in a test must not be weighed or measured after acclimation has begun.

4.5.7 Chemical and physical data

If a freshwater dilution water is used, its hardness, alkalinity, pH and specific conductance should be measured. If a brackish or marine dilution water is used, its salinity and pH should be measured. The dissolved oxygen concentration should be measured at the beginning of the test and every 48 hours thereafter to the end of the test in all test concentrations as long as test organisms are present. The pH should be measured at the beginning and end of the test in all test concentrations. Temperature, salinity or hardness, alkalinity and specific conductance should be recorded daily. (APHA/AWWA/WPCF, 1976)

If possible, the concentration of test material should be measured at the beginning and end of the test in all test chambers, especially in the case of flow-through toxicity tests. When the concentration is not measured, the usefulness of the flow-through tests may be greatly diminished. The analytical method for measuring the concentration of test material must be validated before the beginning of the test. As a minimum, a measure of the accuracy of the method must be obtained by using the method of known additions with dilution water from a tank containing test organisms. On each of two separate days three samples should be analysed at the next to the lowest test concentration that will be used in the toxicity test. The accuracy of standard solutions should be checked against other standard solutions whenever possible. In general, atomic absorption spectrophotometric methods for metals, and gas chromatographic methods for organic test materials are preferable to colorimetric methods.

Whenever samples from a toxicity test are analysed, at least one reagent blank must also be analysed, if appropriate. Also, at least one sample for the method of known additions should be prepared by adding test material to water from a control test chamber to match the next to the lowest test concentration used in the toxicity test.

When water samples are taken from test chambers, they should be taken midway between the top, bottom and sides of the test chambers and should not include any surface scum or material stirred up from the bottom or sides.

4.6 Procedures

4.6.1 Range-finding test

Unless the approximate toxicity of the test material is already known, it is usually desirable to conduct a range-finding test to determine the concentrations of test material that should be used in the definitive test. Generally, groups of five organisms are exposed to three to five widely-spaced toxicant concentrations and a control for 24 to 96

hours using the static or flow-through techniques. The greater the similarity between the range-finding test and the definitive test, the more useful the results of the range-finding test will be.

4.6.2 Definitive test

For the determination of an LC50 or an EC50, a control (and solvent control if necessary) and at least five concentrations of test material in a geometric progression should be used, that is a sequence in which the ratio of a concentration to its predecessor is always the same (10, 5, 2.5, 1.25). More treatments are desirable to ensure the acceptability of the test and to provide additional data for various durations of exposure. A definitive test should meet both of the following criteria so that the LC50 or EC50 can be calculated with reasonable accuracy:

(a) Except for the controls, the concentration of test material in each treatment should be at least 50% of the next higher test concentration.

(b) One treatment other than the control should have killed or affected less than 35% of the organisms exposed to it, and one treatment should have killed or affected more than 65% of the organisms. It is desirable to have several test concentrations which provide >0%<100% mortality or effect.

4.7 Data Analysis and Interpretation

4.7.1 Calculations

For each set of data, the LC50 or EC50 and its 95% confidence limits should be calculated on the basis of the measured initial concentrations of test material for static tests and the average measured concentrations of test material for flow-through tests. If measured concentrations of the test material are not available, the calculated (nominal) initial concentrations should be used. For effluents, the calculated initial volume percent of the effluent in the test solutions should be used. The "volume percent" equals (100 x volume of effluent)/(volume of effluent plus volume of dilution water). A variety of methods can be used to calculate an LC50 or EC50, but the most widely used are the probit (Finney, 1971), logit, moving average, and Litchfield and Wilcoxon (1949) methods. The percentage of the test organisms that die or show the effect in the control treatment must not be used in the calculation of the results. No corrections should be made for control mortality.

According to Stephan (1977), almost all concentration-mortality data produced by acute mortality tests can be analysed to produce a statistical "best estimate" of the LC50 and its 95% confidence limits by using the moving average method and log transformation of the concentration data. The exception is the case in which none of the test concentrations contain test organism mortality >0%<100%. The binomial test can then be used to obtain an estimate of the LC50 as LC50 = $(AB)^{\frac{1}{2}}$, where A = highest test material concentration in which none of the test organisms died and B = lowest concentration in which all the organisms died. Although 95% confidence limits cannot be calculated without test concentrations in which test organism mortality was >0<100% generally they must lie between A and B. The confidence level associated with these limits depends on the number of organisms exposed and can be calculated from the formula: Confidence level = $100\left[1-2\left(\frac{1}{2}\right)^{N}\right]$, where N = number of exposed organisms.

Number of organisms	Confidence level
5	93.8
7	98.4
10	99.9

Thus, if six or more organisms are used per treatment, the confidence level represented by A and B is always above 95%.

4.7.2 Ecological significance of test results

As stated by FAO (1977), any detectable and measurable response of organisms in a toxicity test should not be interpreted as an ecologically significant event. The effect must be related to the expected environmental concentration (Cairns et al., 1978) in order to make a judgement about the risk posed by a pollutant in the environment and thus to estimate its ecological significance.

Indeed, laboratory tests may give conservative estimates of field effects because of the absence of mitigating factors (sunlight, sediment, microbes, etc.) that would decrease the toxicity of the test material under "natural" conditions.

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